AVIAN DISEASES 49:112–117, 2005

Resistance to Intestinal Coccidiosis Following DNA Immunization with the Cloned 3-1E *Eimeria* Gene Plus IL-2, IL-15, and IFN-γ

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Received 30 July 2004; Accepted 21 October 2004

SUMMARY. A cloned *Eimeria acervulina* gene (3-1E) was used to vaccinate chickens *in ovo* against coccidiosis, both alone and in combination with genes encoding interleukin (IL)-1, IL-2, IL-6, IL-8, IL-15, IL-16, IL-17, IL-18, or interferon (IFN)-γ. Vaccination efficacy was assessed by increased serum anti–3-1E antibody titers, reduced fecal oocyst shedding, and enhanced body weight gain following experimental infection with *E. acervulina*. When used alone, anti–3-1E antibody titers were transiently, but reproducibly, increased at 2 wk and 3 wk posthatching in a dose-dependent manner. Similarly, significantly reduced oocyst shedding and increased weight gain were observed at relatively high-dose 3-1E vaccinations (≥25 μg/egg). Combined immunization with the 3-1E and IL-1, IL-2, IL-15, or IFN-γ genes induced higher serum antibody responses compared with immunization with 3-1E alone. Following parasite infection, chickens hatched from embryos given the 3-1E gene plus the IL-2 or IL-15 genes displayed significantly reduced oocyst shedding compared with those given 3-1E alone, while 3-1E plus IL-15 or IFN-γ significantly increased weight gain compared with administration of 3-1E alone. Taken together, these results indicate that *in ovo* immunization with a recombinant *Eimeria* gene in conjunction with cytokine adjuvants stimulates protective intestinal immunity against coccidiosis.

RESUMEN. Resistencia a la coccidiosis intestinal posterior a la inmunización con ADN con los genes clonados 3-1E de *Eimeria* y los genes de IL-2, IL-15 e IFN-γ.

Se vacunaron pollos *in ovo* contra la coccidiosis empleando el gen clonado 3-1E de *Eimeria acervulina* solo o en combinación con genes que codifican las interleuquinas 1 (IL-1), IL-2, IL-6, IL-8, IL-15, IL-16, IL-17, IL-18, o interferón gamma (IFN-γ). Se determinó la eficacia de la vacunación mediante el incremento de los títulos de anticuerpos contra el gen 3-1E, la reducción de la excreción de oocistos en las heces, y el incremento en el peso corporal posterior a la infección experimental con *E. acervulina*. Al emplear solo el gen 3-1E, se observaron títulos de anticuerpos transitorios pero reproducibles, los cuales aumentaron a las 2 y 3 semanas posteriores al nacimiento dependiendo de la dosis empleada. Igualmente se observó una reducción significativa en el número de oocistos excretados en las heces y un incremento significativo en la ganancia de peso en las vacunaciones empleando dosis relativamente altas (≥ 25 μg/huevo). La vacunación combinada empleando el gen 3-1E en asociación con los genes IL-1, IL-2, IL-15, e IFN-γ, indujo una mayor respuesta de anticuerpos al ser comparada con la inmunización empleando únicamente el gen 3-1E. Posterior a la infección con coccidia, los pollitos provenientes de embriones inoculados con el gen 3-1E en asociación con la IL-2 o la IL-15 mostraron una reducción significativa en la excreción de oocistos al ser comparada con los pollitos provenientes de embriones inoculados únicamente con el gen 3-1E. Se observó un incremento significativo en la ganancia de peso en los pollos provenientes de embriones inoculados con el gen 3-1E en asociación con el gen IL-15 o el gen IFN-γ al ser comparado con el observado en pollitos provenientes de embriones inoculados únicamente con el gen 3-1E. Los resultados obtenidos indican que la inmunización *in ovo* con la vacuna recombinante que contiene el gen de *Eimeria* en asociación con citoquinas adyuvantes estimulan la inmunidad intestinal protectora contra la coccidiosis.

Key words: chicken, cytokine, immunity, parasite, *in ovo* vaccination, coccidiosis

Abbreviations: BSA = bovine serum albumin; ELISA = enzyme-linked immunosorbent assay; IFN = interferon; IgG = immunoglobulin G; IL = interleukin; OD_{450} = optical density at 450 nm; PBS = phosphate-buffered saline; SD = standard deviation; Th1 = T-helper 1

Fifteen years ago, Wolff and colleagues (46) demonstrated that administration of naked DNA—in the form of a plasmid containing a cloned gene—to the skeletal muscle of mice led to expression of the introduced gene product. Following their discovery, it was demonstrated that the expressed foreign proteins retained native immunogenicity and stimulated antibody and cellular immune responses (40). Use of DNA vaccines for treatment of infectious diseases represents a novel approach in human and veterinary medicine. In the case of avian coccidiosis, an economically important disease that seriously impairs the feed utilization and growth of commercial poultry (1,16), immunization with recombinant genes cloned from the genome of *Eimeria* spp. has been shown to offer protection against development of clinical disease when delivered orally, subcutaneously, or intramuscularly (9,12,13).

Over the past two decades, an alternative route of poultry vaccination—embryo injection—has emerged as a safe, efficient, and automated method to deliver immunogens (11,32). Early studies by Sharma and coworkers (36,37,38,39) demonstrated that *in ovo* vaccination induced resistance to a variety of important viral diseases. More recently, *in ovo* administration of avian vaccines has been described both in experimental and industrial settings against an expanded repertoire of infectious agents (22,31). Commercially, the advantages offered by *in ovo* vaccination include early immunity, reduced bird stress, precise and uniform dosaging, multiple-agent delivery, ease of handling, and reduced labor costs. Over seven billion broilers produced annually in the United States are inoculated *in ovo* (2).

Although embryo vaccination has been used to control infectious diseases due to viral pathogens, it has proved less successful for other microorganisms, including those responsible for parasitic infections such as cryptosporidiosis and coccidiosis (8,43,44). For less immunogenic vaccines, co-administration of cytokines often potentiates subsequent acquired immunity (3,17,20,21,27). For example, recombinant interleukin (IL)-5 and IL-6 markedly increased antibody

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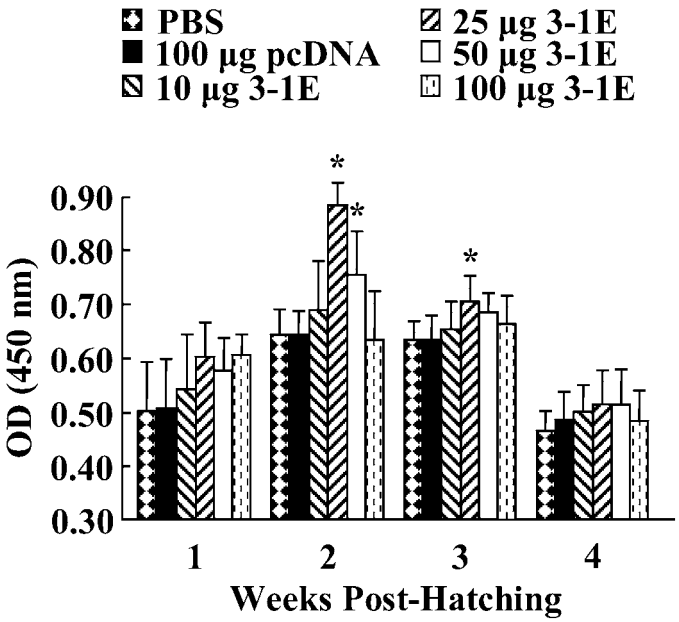

Fig. 1. Serum antibody levels following *in ovo* vaccination with the 3-1E gene. Chickens (20/group) were vaccinated *in ovo* with 100 µl of PBS, 100 µg of the pcDNA empty vector, or 10, 25, 50, or 100 µg of 3-1E–pcDNA at day 18 of embryonation. At 1, 2, 3, and 4 wk posthatching, blood was collected by cardiac puncture and anti–3-1E serum antibody levels determined by ELISA. Each bar represents the mean ± SD ($N = 5$). Asterisks (*) indicate significantly increased OD_{450} values compared with the pcDNA-only group for the respective time points ($P < 0.05$).

levels to coexpressed heterologous antigens in mice (29). Pasquini *et al.* (28) showed that a variety of cytokines, including IL-2, IL-4, IL-12, and interferon (IFN)-γ, amplified parameters of immunity to genetic vaccines. Studies from our laboratory (14,15,25) as well as others (19,20) demonstrated that chicken IL-2 and IFN-γ enhanced protective immunity to experimental coccidiosis. In the study reported here, a cloned *Eimeria* gene (3-1E) was evaluated alone and in combination with a panel of nine recombinant cytokine genes in an *in ovo* vaccination trial to stimulate resistance to intestinal coccidiosis. We observed significantly reduced fecal oocyst shedding and increased body weight gain, indicators of diminished disease severity, following DNA embryo vaccination.

MATERIALS AND METHODS

Materials. All materials were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated.

Cloning of the 3-1E gene. The 3-1E gene was originally cloned by immunoscreening an *Eimeria acervulina* cDNA library with a rabbit antiserum against *E. acervulina* merozoites (15). The 1086–base pair 3-1E cDNA was subcloned into the pcDNA expression vector (Invitrogen, Carlsbad, CA), as described (41). The recombinant 3-1E–pcDNA plasmid was transformed into *Escherichia coli* DH5α, bacteria were grown overnight to mid-log phase, and plasmid DNA was purified using a commercial kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. DNA was quantified spectrophotometrically.

Chicken cytokine genes. cDNAs encoding IL-2, IL-6, IL-8, IL-15, IL-16, IL-17, and IFN-γ, all cloned in the pcDNA vector, have been described (5,15,23,24,25,42). pcDNA expression plasmids encoding IL-1 (45) and IL-18 (35) were generously provided by Dr. Peter Staeheli (University of Freiberg, Germany). All plasmids were purified as described above.

***In ovo* immunization.** Eggs of specific-pathogen-free white Leghorn SC inbred chickens (HyLine International, Dallas Center, IA) were incubated to day 18 of embryonation, candled to select fertile eggs, and injected with the pcDNA empty vector or the 3-1E–pcDNA recombinant plasmid either alone or in combination with cytokine genes, as described (36). Briefly, plasmid DNAs in 100 µl of sterile phosphate-buffered saline (PBS), pH 7.4, were injected into the amniotic sac by the entire length of a 3.1-cm, 22-gauge needle using an Intelliject instrument (Avitech, Easton, MD). Hatching rates for the uninjected control and injected eggs were 92%–100%. All experiments were performed according to guidelines established by the Beltsville Agriculture Research Center Small Animal Care Committee.

Experimental infections. The wild-type strain of *E. acervulina*, originally developed at the Animal and Natural Resources Institute (Beltsville, MD), was used for oral inoculations. Oocysts were cleaned by floatation on 5.25% sodium hypochloride, washed three times with PBS, and enumerated with a hemocytometer. Chickens were infected with 10,000 oocysts, as described (18) at day 14 posthatching. Body weights were measured at day 1 preinfection and day 5 postinfection. Fecal samples were collected individually from each chicken between days 5 and 10 postinfection, and the number of oocysts were determined as described (18).

Anti–3-1E antibody enzyme-linked immunosorbent assay (ELISA). The 3-1E protein was isolated and its purity confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting, as described (15,41). Flat-bottom, 96-well microtiter plates (Costar, Boston, MA) were coated with 100 µl of purified 3-1E protein (10 µg/ml) in 0.1 M sodium carbonated buffer, pH 9.6, at 4 C overnight. Wells were blocked with 100 µl of PBS containing 1% bovine serum albumin (BSA) for 1 hr at room temperature and incubated with 100 µl of serum samples for 2 hr at room temperature. The wells were washed five times with PBS containing 0.05% Tween 20 and incubated for 30 min at room temperature with 100 µl of horseradish peroxidase–conjugated anti-chicken immunoglobulin G (IgG) diluted 1:4000 in PBS–1% BSA. The wells were washed five times, developed with 100 µl of 0.01% tetramethylbenzidine in 0.05 M phosphate–citrate buffer, pH 5.0, for 10 min, followed by 50 µl of 2 N H_2SO_4, and optical densities at 450 nm (OD_{450}) were measured.

Statistical analyses. Each experimental group consisted of five replicates. Mean and standard deviation (SD) values for antibody levels and body weight gains were compared by the Yuker–Kramer Multiple Comparisons test. Mean ± SD values for fecal oocyst shedding were compared by the Dunnett Multiple Comparisons test. Differences between means were considered significant at $P < 0.05$.

RESULTS

Anti–3-1E antibody levels following *in ovo* vaccination with the 3-1E gene. To assess serum antibody levels against the 3-1E protein following *in ovo* injection of the 3-1E gene, 120 fertile eggs were divided into six groups (20/group) and vaccinated with 100 µl of PBS, 100 µg of the pcDNA empty vector, or 10, 25, 50, or 100 µg of the 3-1E gene. At 1, 2, 3, and 4 wk posthatching, five chickens per group were bled by cardiac puncture, and the levels of anti–3-1E antibodies were measured by ELISA. As shown in Fig. 1, antibodies were first detected at 2 wk posthatching in the 25- and 50-µg dose groups, but rapidly decreased thereafter. Although the observed humoral immune response following 3-1E DNA embryo immunization was transient, these results were repeated in three separate vaccination trials.

Protective immunity to coccidiosis following *in ovo* vaccination with the 3-1E gene. To assess the effects of *in ovo* vaccination with the 3-1E DNA on *E. acervulina* infection, 140 fertile eggs were divided into seven groups (20/group) and vaccinated with 100 µl of PBS, 50 µg of the pcDNA empty vector, or 1, 5, 10, 25, or

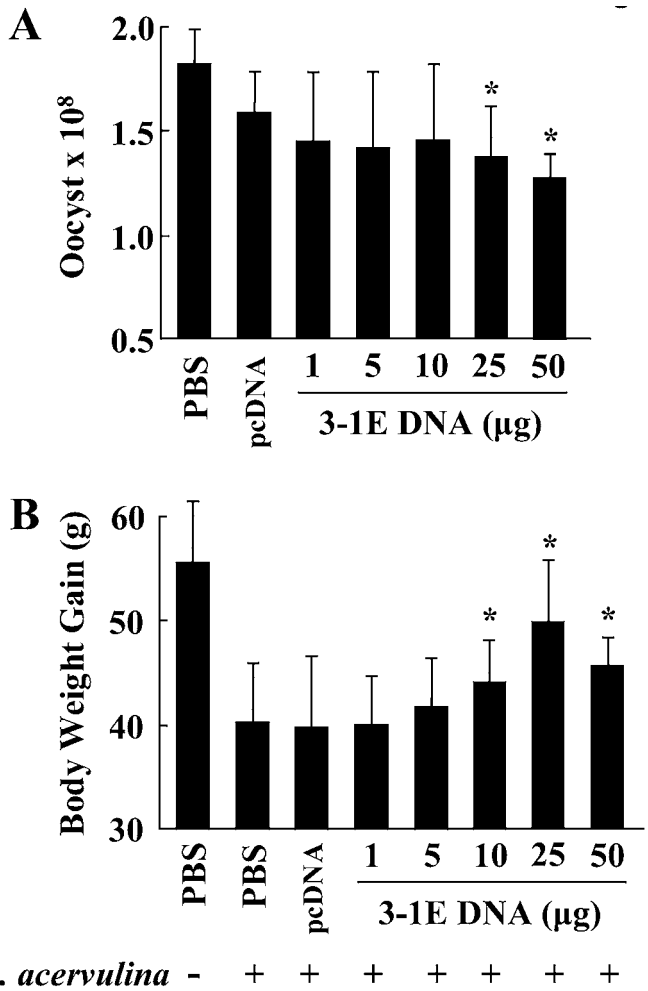

Fig. 2. Protective immunity following *in ovo* vaccination with the 3-1E gene. (A) Chickens (20/group) were vaccinated *in ovo* with 100 μl of PBS, 100 μg of the pcDNA empty vector, or 1, 5, 10, 25, or 50 μg of 3-1E–pcDNA at day 18 of embryonation. At day 14 posthatching, chickens were orally infected with 10,000 sporulated oocysts of *E. acervulina* and fecal oocyst shedding determined between days 5 and 10 postinfection. (B) Body weight gains were determined between day 1 preinfection and day 5 postinfection. Each bar represents the mean ± SD ($N = 10$). Asterisks (*) indicate significantly decreased oocyst shedding or increased weight gain compared with the pcDNA-only groups ($P < 0.05$).

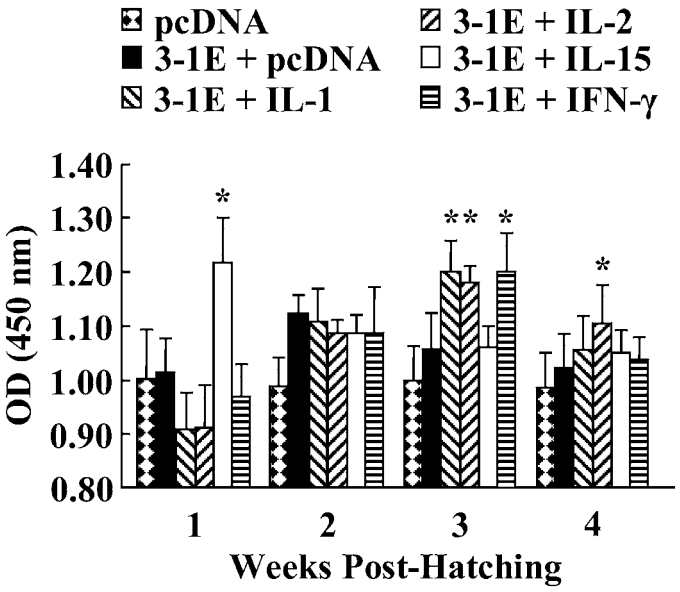

Fig. 3. Serum antibody levels following *in ovo* covaccination with 3-1E and cytokine genes. Chickens (20/group) were vaccinated *in ovo* with 50 μg of the pcDNA empty vector or 25 μg of 3-1E–pcDNA plus 25 μg of pcDNA empty vector or pcDNA encoding IL-1, IL-2, IL-15, or IFN-γ at day 18 of embryonation. At 1, 2, 3, and 4 wk posthatching, blood was collected by cardiac puncture and anti–3-1E serum antibody levels determined by ELISA. Each bar represents the mean ± SD ($N = 5$). Crosses (+) indicate significantly increased OD_{450} values compared with the pcDNA-only group for the respective time points ($P < 0.05$). Asterisks (*) indicate significantly increased OD_{450} values compared with the 3-1E–pcDNA plus pcDNA group for the respective time points ($P < 0.05$).

50 μg of the 3-1E gene. At day 14 posthatching, all chickens were orally infected with 10,000 sporulated oocysts of *E. acervulina*, and fecal oocyst shedding was determined between days 5 and 10 postinfection. As shown in Fig. 2A, significantly decreased oocyst shedding was observed following injection of the 25- and 50-μg 3-1E DNA groups compared with the pcDNA vector–only group ($P < 0.05$). In a second vaccination trial, eight groups of eggs (20/group) were injected with PBS (groups 1 and 2), pcDNA (group 3), or 3-1E–pcDNA (groups 4–8) as above, either noninfected (group 1) or infected with *E. acervulina* at day 14 posthatching (groups 2–8), and body weights were determined at day 1 preinfection and day 5 postinfection. As shown in Fig. 2B, significantly increased weight gains were seen following vaccination with 10, 25, or 50 μg of 3-1E DNA, compared with the pcDNA empty-vector group ($P < 0.05$). Taken together, these results indicated that *in ovo* vaccination with the 3-1E gene induced partial protection against experimental coccidiosis.

Effects of *in ovo* covaccination with 3-1E and cytokine genes. To investigate the ability of chicken cytokine genes to augment the effects of 3-1E gene immunization, we next analyzed anti–3-1E antibody responses, fecal oocyst shedding, and weight gains of infected chickens following embryo covaccination of 3-1E with a panel of nine different cytokines (IL-1, IL-2, IL-6, IL-8, IL-15, IL-16, IL-17, IL-18, and IFN-γ). To determine the effects on antibody levels, 120 eggs were divided into six groups, and 20 eggs/group were vaccinated with 50 μg of the pcDNA empty vector or 25 μg of the 3-1E–pcDNA plasmid plus 25 μg of pcDNA alone or pcDNA encoding IL-1, IL-2, IL-15, or IFN-γ. As was the case with the results presented in Fig. 1, anti–3-1E antibody titers were significantly increased at weeks 2 and 3 posthatching following immunization with 3-1E DNA alone compared with the pcDNA empty vector (Fig. 3). Moreover, antibody levels were further increased at 1 wk posthatching following vaccination with the 3-1E and IL-15 genes and at 3 wk following immunization with 3-1E plus IL-1, IL-2, or IFN-γ genes, compared with those immunized with 3-1E alone. At 4 wk posthatching, antibody levels following *in ovo* vaccination with 3-1E plus IL-2 remained significantly increased compared with the results for the control group that received the pcDNA empty vector. Covaccination of 3-1E plus IL-6, IL-8, IL-16, IL-17, or IL-18 did not boost anti–3-1E antibody titers above that achieved with 3-1E alone (data not shown).

To determine the effects of 3-1E/cytokine gene *in ovo* covaccination on resistance to coccidiosis, 11 groups (20/group) were vaccinated with 50 μg of pcDNA alone (group 1) or 25 μg of 3-1E–pcDNA plus 25 μg of the pcDNA empty vector or one of the nine different cytokine genes (groups 2–11). At day 14 posthatching,

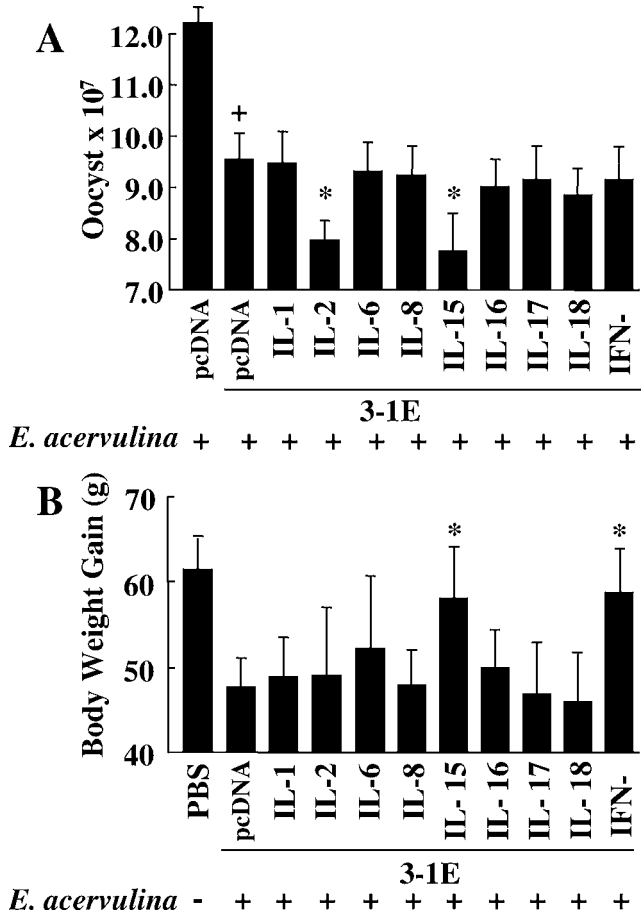

Fig. 4. Protective immunity following *in ovo* covaccination with 3-1E and cytokine genes. Chickens (20/group) were vaccinated *in ovo* with 100 μl of PBS, 50 ug of the pcDNA empty vector, or 25 μg of 3-1E–pcDNA with 25 μg of pcDNA carrying chicken cytokine gene at day 18 of embryonation. At day 14 posthatching, chickens were orally infected with 10,000 sporulated oocysts of *E. acervulina* and fecal oocyst shedding determined between days 5 and 10 postinfection (A). Body weight gains (10/group) were determined between day 1 preinfection and day 5 postinfection (B). Each bar represents the mean ± SD ($N = 10$). Crosses (+) indicate significantly decreased oocyst shedding compared with the pcDNA-only group ($P < 0.05$). Asterisks (*) indicate significantly decreased oocyst shedding or increased weight gain compared with the 3-1E–pcDNA plus pcDNA groups ($P < 0.05$).

all chickens were orally infected with 10,000 sporulated oocysts of *E. acervulina* and fecal oocyst shedding was determined between days 5 and 10 postinfection. As shown in Fig. 4A, embryo immunization with 3-1E alone decreased oocyst shedding approximately 20% compared with the pcDNA empty vector–injected group, a result consistent with the data presented in Fig. 2A. In addition, vaccination with 3-1E plus the IL-2 or IL-15 DNAs further decreased oocyst shedding compared with vaccination with 3-1E alone ($P < 0.05$). Body weights were determined at day 1 preinfection and day 5 postinfection. As shown in Fig. 4B, *in ovo* injection of the 3-1E and IL-15 or IFN-γ genes completely reversed the weight gain loss produced by *E. acervulina* infection. That is, whereas vaccination with 3-1E alone led to approximately 17% loss of weight gain *vs.* that observed in noninfected controls (compared with approximately 30% loss in the nonvaccinated, infected group; Fig. 2B), the differences between 3-1E plus IL-15 or IFN-γ groups and the noninfected group were not statistically significant ($P > 0.05$).

DISCUSSION

The data presented in this report are summarized as follows: i) *in ovo* vaccination with the *Eimeria* 3-1E gene generated an antibody response against the expressed parasite protein that was enhanced by covaccination with the IL-1, IL-2, IL-15, or IFN-γ genes; ii) vaccination with 3-1E augmented protective immunity against *E. acervulina* infection, as measured by reduced fecal oocyst shedding and increased body weight gain compared with that observed in nonvaccinated controls; and iii) covaccination with 3-1E plus the IL-2, IL-15, or IFN-γ genes led to further reduction of oocyst shedding and increased weight gain beyond that induced by 3-1E alone.

Our laboratory first cloned and characterized the 3-1E gene and its corresponding protein as potential vaccines for coccidiosis (15). The 3-1E gene encodes a 23-kD polypeptide highly conserved among various life-cycle stages and species of *Eimeria*. Antibodies produced against the recombinant 3-1E protein reacted with sporozoites and merozoites of *E. acervulina*, *E. tenella*, and *E. maxima*. Furthermore, we showed that spleen lymphocytes from *E. acervulina*–immunized chickens exhibited antigen-specific proliferation and IFN-γ production upon stimulation with the recombinant 3-1E protein, indicating activated cell-mediated immunity during coccidiosis. In addition, significantly reduced oocyst shedding by *E. acervulina*–infected chickens was observed following intramuscular or subcutaneous immunization with the 3-1E–pcDNA plasmid (41). In the current study, we have confirmed and extended these results by showing that *in ovo* gene vaccination with 3-1E, alone or in combination with cytokine genes, induced protection against experimental coccidiosis.

In mammalian systems, a variety of different experimental parameters affect the efficacy of naked DNA immunization. In general, the route of DNA inoculation dictates the type of immune response elicited (e.g., T-helper 1 [Th1] *vs.* Th2). Generally, intramuscular and intradermal injections stimulated Th1 responses characterized by increased production of IFN-γ and antigen specific IgG2a antibody. In contrast, epidermal injection elicited Th2 responses manifested by elevated levels of IL-4 and IgG1 antibody (33). Other parameters of variability include the dose and vaccination timing with respect to coadministration of adjuvants (48) and potential nonspecific effects of nonmethylated CpG dinucleotides in plasmid vectors (6,34). In the experiments reported here, chickens injected with the pcDNA3 plasmid alone did not show statistically significant changes in anti–3-1E antibody levels, weight gain, or fecal oocyst shedding, thus indicating that the effects produced by DNA vaccination were not attributed to nonspecific factors related to the plasmid DNA.

Cytokines are potent adjuvants that modulate host immunity to infectious diseases (3,27). When used alone, cytokines have been shown to improve humoral and cellular immune responses. In chickens, for example, subcutaneous injection of a recombinant plasmid encoding the IL-2 gene enhanced T-cell and natural killer cell activities *in vitro* (4). We previously reported that recombinant IFN-γ blocked *E. tenella* development *in vitro* and reduced fecal oocyst shedding and body weight loss following *E. acervulina* challenge infection (14). Subsequently it was demonstrated that analogous effects were manifested by vaccination with a cDNA encoding the IFN-γ gene (25). Authors of other work (10,17,19,20) also observed that chickens treated with recombinant IFN-γ showed increased weight gains compared with untreated controls and exhibited smaller weight gain reductions upon challenge with

Eimeria parasites. Heriveau *et al.* (7) reported that chicken macrophages, fibroblasts, and epithelial cells pretreated with recombinant IFN-γ inhibited *E. tenella* replication *in vitro.* A similar effect was not observed using chicken IFN-α.

Cytokines in combination with vaccines often are capable of enhancing protective immunity to a degree greater than that achieved by use of the vaccine alone (17). For example, we (25) showed that subcutaneous vaccination with the 3-1E gene in combination with plasmids encoding the IFN-α or lymphotactin genes significantly protected chickens against body weight loss induced by *E. acervulina* infection. In addition, parasite replication was significantly reduced in chickens given the 3-1E–pcDNA vaccine along with the IL-1, IL-8, IL-15, IFN-γ, transforming growth factor–β4, or lymphotactin genes compared with 3-1E alone. Coadministration of IFN-γ with an experimental immunogen (sheep red blood cells) in chickens resulted in elevated antibody responses relative to the blood cells alone (21). Compared with cows given a live bovine herpes virus vaccine, Reddy *et al.* (30) observed improved virus-specific immunity in animals given the virus in combination with bovine IL-1. Nobiron *et al.* (26) reported that coadministration of IL-2 enhanced antigen-specific immune responses following vaccination with DNA encoding the bovine viral diarrhea virus glycoprotein E2. Similarly, a DNA vaccine against foot-and-mouth disease elicited immunity in swine that was enhanced by coadministration with IL-2 (47).

In view of the potentially beneficial effects of chicken cytokine as a nonspecific immunomodulator, current studies in our laboratory are directed at further evaluating the potential use for cytokine genes and gene products as coccidiosis vaccine adjuvants.

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ACKNOWLEDGMENTS

We are thankful for suggestions provided by Dr. Wongi Min and Dr. Rami Dalloul and the technical expertise of Ms. Diane Hawkins and Ms. Margie Nichols. This project was supported by National Research Initiative grant 2004-35204-14798 from the CSREES of the United States Department of Agriculture.